AD_____

Award Number: DAMD17-98-1-8297

TITLE: Signaling by ErbB Receptors in Breast Cancer: Regulation by Compartmentalization of Heterodimetric Receptor Complexes

PRINCIPAL INVESTIGATOR: Andrea H. Bild
Gary Johnson

CONTRACTING ORGANIZATION: University of Colorado Health Sciences Center
Denver, Colorado 80262

REPORT DATE: October 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2001	Annual Summary (15 Sep 98 - 14 Sep 01)

4. TITLE AND SUBTITLE
Signaling by ErbB Receptors in Breast Cancer: Regulation by Compartmentalization of Heterodimetric Receptor Complexes

5. FUNDING NUMBERS
DAMD17-98-1-8297

6. AUTHOR(S)
Andrea H. Bild
Gary Johnson

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Colorado Health Sciences Center
Denver, Colorado 80262

E-Mail: Andrea.Bild@uchsc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Herein, we investigated the mechanisms of MEKK1 and Stat3 signaling pathways. MEKK1 is important in apoptosis of cancer cells following detachment of cells from their matrix and following genotoxin treatment. We determined the mode by which MEKK1 causes apoptosis, and the ability of specific anti-apoptotic pathways to circumvent MEKK1-induced apoptosis. Specifically, MEKK1-induced apoptosis requires cleavage by caspase 3 and activation of the TRAIL death receptor pathway. MEKK1-induced death is potentiated by inhibition of PI-3 kinase, and is blocked by Akt through inhibition of MEKK1 cleavage. We also examined the mode of Stat3 transport through the cytoplasm to the nucleus, which has remained elusive. Stat3 is an oncogene implicated in cancer cell survival. We report that the mechanism whereby Stat3 is transported through the cytoplasm involves receptor-mediated endocytosis. Disruption of endocytosis with specific inhibitors blocks Stat3 nuclear translocation and Stat3-dependent gene regulation. Inhibition of endocytosis also blocks IL-6 and v-Src induced Stat3 activation. Therefore, receptor-mediated endocytosis may be a general mechanism for transport of cytoplasmic signaling proteins to the nucleus.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
29

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION:

The mechanisms of intracellular signal transduction are becoming increasingly important to understanding the basis of disease. In complex signaling pathways, proteins have the ability to cause either cell survival or apoptosis depending on factors such as stimuli, subcellular localization and the presence of particular co-factors that may depend on cell type. Therefore, it is important not only to understand the upstream and downstream effects of a signaling protein but to also comprehend the mechanism by which it carries out its role. The understanding of the mechanism by which a protein functions will allow design of drugs not just targeted at a basic function, such as blocking DNA synthesis, but at the specific mechanism by which a protein carries out its effect, such as phosphorylation or endocytosis.

One of the main mechanisms by which proteins signal from extracellular stimuli at the cell membrane to the nucleus and ultimately physiological effects is via kinase activation. Mitogen-Activated Protein Kinase (MAPK) is a signal transduction pathway activated in response to a diverse array of stimuli at the cell surface which vary from growth factors and cytokines to osmolarity and shear stress. MEK kinase 1 (MEKK1) is a 196-kDa mitogen-activated protein kinase (MAPK) kinase kinase that participates in regulation of the c-Jun N-terminal kinase (JNK) and extracellular signal regulated kinase (ERK) pathways (1,2). MEKK1 is also involved in induction of apoptosis in response to a wide variety of stimuli [Widmann, 1997 #17; Widmann, 1999 #84]. In contrast to the sequential MAPK cascade, there are pathways which contain transcription factors such as STAT and Smad that signal directly from receptors at the cell membrane to the nucleus. STATs are unique among signaling proteins in that they are nuclear transcription factors activated by receptors at the cell membrane, thereby directly converting stimuli at the cell surface to regulation of gene transcription. Stat3 is found to be activated in many cancer cells. To understand the mechanism by which such diverse signaling pathways carry out their effect, I studied the mode by which MEKK1 signals for apoptosis and the mechanism by which Stat3 translocates to the nucleus.

BODY:

Mechanism of MEKK1 and Stat3 Regulation in Signaling Pathways Involved in Breast Cancer Cell Survival

Task 1. To identify the mechanism by which MEKK1 causes apoptosis, and the ability of the Akt oncogene to subvert this apoptotic pathway.

Herein, we investigate the mode by which MEKK1 causes apoptosis, and the ability of specific anti-apoptotic pathways to circumvent MEKK1-induced apoptosis. MEK kinase 1 (MEKK1) is a 196-kDa mitogen-activated protein kinase (MAPK) kinase kinase that participates in regulation of the c-Jun N-terminal kinase (JNK) and extracellular signal regulated kinase (ERK) pathways (1,2). MEKK1 is also involved in induction of apoptosis through the activation of caspases [Widmann, 1997 #17; Widmann, 1999 #84]. Exposure of cells to stresses, such as genotoxins, activates caspase 3-like proteases which cleave MEKK1 into a pro-apoptotic 91-kDa kinase domain fragment (3). This cleavage of MEKK1 releases its 91 kDa form from the membrane fractions into the cytoplasm (4,5). The 91-kDa kinase fragment further amplifies caspase activation in a feedback loop and is a strong inducer of apoptosis (3,6). Inhibitors of caspases block this cleavage, and mutation of the consensus caspase 3-like cleavage site of MEKK1 inhibits both its cleavage and its ability to induce apoptosis. Both cleavage of MEKK1 and increased expression of death receptor 4 (DR4, TRAILR1) and DR5 (TRAILR2) occur following exposure of cells to genotoxins (7). Overexpression of a kinase inactive MEKK1 inhibits MEKK1-mediated apoptosis and effectively blocks death receptor upregulation following etoposide treatment (7). Thus, cleavage of MEKK1 serves to activate a cell death-promoting response.

MEKK1 is emerging as an important mediator of apoptosis in human cancers. MEKK1 is involved in apoptosis of colon cancer cells following chemotherapy (8). Furthermore, aberrant MEKK1 cleavage and subsequent apoptosis in certain ovarian adenocarcinomas leads to drug resistance (9). Dysregulation of apoptosis also occurs by mutation of genes involved in the death receptor pathways. Death receptors such as DR4, DR5, Fas, and tumor necrosis factor (TNF) receptor belong to the superfamily of TNF receptors that initiate apoptotic signals upon ligation. Activation of these death receptors leads to recruitment of other proteins, including FADD (an adaptor protein) and caspase 8. Association of adaptor proteins with the death receptor leads to caspase 8 activation, which in turn leads to the activation of caspase 3-like molecules and eventual apoptotic cell death (10-12). Inactivating mutations in death receptor 4 (DR4, TRAIL-R1) and death receptor 5 (DR5, TRAIL-R2) have been characterized in certain metastatic breast cancer and non-Hodgkin's lymphomas (13,14). Further Fas mutations occur in specific malignant lymphomas and solid tumors (15,16). Other TNF receptor family members, such as decoy receptor 1 (DcR1), bind to ligands for death receptors but, lacking the cytoplasmic domains to recruit pro-apoptotic proteins, fail to induce apoptosis (17). By competitive inhibition, these decoy receptors act to circumvent death receptor activation, thus negatively regulating death receptor-induced apoptosis.

Another protein which negatively regulates apoptosis is the Akt proto-oncogene. Akt is found upregulated in many cancers, including breast, ovarian, prostate, and pancreatic malignancies (18,19). Akt mediates cell survival by suppressing apoptosis induced by a variety of apoptotic stimuli, including loss of cell adhesion (anoikis), growth factor withdrawal, and exposure to genotoxins. The action of Akt-mediated protection occurs both by inhibition of pro-apoptotic proteins, such as caspase 9, BAD, and the Forkhead transcription factor, and by activation of anti-apoptotic proteins, such as the NF-κB and CREB. Akt can also promote cell survival through inactivation of caspase-mediated apoptotic signaling (20,21). Recent studies suggest a role of Akt in inhibitory modulation of the TNF-receptor mediated apoptosis. Akt is also implicated in Fas-mediated apoptosis as Pten +/- mice show decreased Fas-induced apoptosis (22). Also, Akt regulates the expression of c-FLIP, a caspase 8 dominant negative which inhibits Fas death signals, in certain tumor cells (23,24).

To further define the mechanism of MEKK1-mediated apoptosis, we investigated the role TRAIL receptor pathway activation. Transfection studies involving overexpression of DcR1 and dominant negative FADD (FADD DN) show that death receptor signaling is required for MEKK1-mediated apoptosis. Inhibition of PI-3 kinase potentiates MEKK1-induced apoptosis, and expression of Akt blocks initial cleavage of endogenous MEKK1 to its pro-apoptotic 91 kDa form and subsequent MEKK1 caspase activation and apoptosis. Thus, MEKK1-induced apoptosis requires death receptor activation and is blocked by Akt through inhibition of caspase activation, which inhibits cleavage of MEKK1 to its pro-apoptotic 91kDa form.

Inhibition of death receptor activation prevents MEKK1-induced apoptosis

Decoy receptors compete with specific death receptors for ligand binding (10). Overexpression of DcR1 blocks ligand binding and activation of DR4 and DR5 (17,25). To determine if DR4 and DR5 activation is involved in MEKK1-induced apoptosis, Human embryonic kidney (HEK) 293 cells overexpressing MEKK1 were transfected with vector alone or with DcR1 cDNA. The extent of apoptosis was determined by TdT staining in a Tunel assay, as described in the Materials and Methods section. HEK 293 cells containing empty vector showed 21% apoptosis when MEKK1 was expressed, compared with 4% apoptosis in cells expressing DcR1 in the presence of MEKK1 (Figure 1A). HEK 293 cells were also transiently transfected with MEKK1 in the absence or presence of FADD DN. FADD DN acts to block caspase 8 activation following ligand binding of death receptors, including DR4 and DR5. Expression of MEKK1 alone caused 43% apoptosis. In the presence of FADD DN, however, MEKK1 induced only 20% apoptosis, a percentage similar to that seen in untransfected cells (Figure 1B). Cells expressing FADD DN without MEKK1 also showed no increase in apoptosis above control levels. These results suggest that MEKK1-induced apoptosis involves activation of TRAIL death receptor pathways.

MEKK1 upregulates DR4 and Fas mRNA and protein levels

To determine if MEKK1 directly affects the mRNA expression levels of TNF death receptor pathway members, RPA analysis was performed. HEK293 cells expressing the 91 kDa MEKK1 kinase domain were lysed and RNA was extracted. RPA analysis using the hApo3d panel was performed [COMPANY]. Cells transfected with 91 kDa MEKK1 show a 2.7-fold increase in Fas and a 1.7-fold increase in DR4 mRNA levels when compared to cells expressing a vector control (Figure 2A-B). Western blot analysis confirms protein increases in Fas and DR4 (data not shown). Therefore, MEKK1 leads to upregulation of death receptors crucial to TNF death receptor pathway.

Inhibition of PI-3 kinase potentiates MEKK1-induced apoptosis

In order to further define the regulation of MEKK1-induced apoptosis, PI-3 kinase activity was inhibited, and the ability of MEKK1 to promote apoptosis was examined. PI-3 kinase is important in cellular survival and has been found to activate Akt and mediate protection of cell from apoptosis. HEK293 cells were treated with 200 nM of a PI-3 kinase inhibitor, wortmannin, for 12 h, and apoptosis was determined as described. By inhibition of endogenous PI-3 kinase, apoptosis induced by overexpression of MEKK1 was potentiated by 24 percent (Figure 3). This result suggests that even basal levels of PI-3 kinase activity can negatively regulate MEKK1-induced apoptosis.

Akt inhibits cleavage of endogenous MEKK1, which requires caspase 3 proteases

Endogenous MEKK1 is cleaved by caspase 3-like proteases following treatment with genotoxic agents. HEK 293 cells stably expressing myr-Akt or vector alone were treated with etoposide (100μM) or UV-C (40 J/m^2). Following treatment, cells were lysed and assayed for full-length MEKK1 by western blotting. 48 hours following etoposide treatment, cleavage of endogenous MEKK1 was inhibited in the presence of a constitutively active myristoylated Akt (myr-Akt) when compared to cells expressing vector alone (Figure 4A). Following UV irradiation, inhibition of cleavage of full length MEKK1 was also seen in cells expressing myr-Akt and wild type Akt (wt-Akt) as compared with cells expressing pCMV5 or β-gal alone (Figure 4B). These results show that Akt over-expression blocks initial cleavage of endogenous MEKK1 to its pro-apoptotic 91

kDa kinase domain fragment. To further define the specific caspase involved in MEKK1 cleavage, we investigated primary mouse embryonic fibroblasts (MEFs) with homozygous deletion of caspase 3. MEF caspase 3-/- cells were treated with etoposide for 24 and 48 hours, and Western blotting was performed to detect the presence of uncleaved, endogenous full-length MEKK1. Cells lacking caspase 3 show no cleavage of full-length MEKK1, while wild type control cells show MEKK1 cleavage (data not shown). Thus, cleavage of MEKK1 into its pro-apoptotic 91 kDa domain specifically requires caspase 3 proteases.

Akt blocks MEKK1-induced apoptosis and caspase 3-like protease activation

Akt is activated by PI-3 kinase and blocks genotoxin-induced apoptosis (21). Since MEKK1 is involved in genotoxin induced apoptosis, the ability of Akt to block MEKK1-induced apoptosis was examined. HEK293 cells were transiently transfected with full length MEKK1 in the presence or absence of myr-Akt, wt-Akt, p35, or vector alone and stained for protein expression using anti-MEKK1 and anti-Akt antibodies. p35 is a protein known to inhibit MEKK1 cleavage and apoptosis and thus acts as a control. Expression of myr-Akt and wt-Akt resulted in increased Akt kinase activity as determined by an *in vitro* Akt kinase assay (data not shown). Percent apoptosis was quantified using a TdT-based TUNEL assay. The number of cells expressing MEKK1 and staining positively for TdT were then counted by fluorescence microscopy. At least 400 cells were counted for each condition in three separate experiments. 42.5% of cells expressing MEKK1 with pCMV5 empty vector were apoptotic. In cells co-expressing MEKK1 with either myr-Akt or wt-Akt, however, the percentage of apoptotic cells was 8.5% and 7.6%, respectively. These results reflect an 80% inhibition of MEKK1-induced apoptosis by Akt. As an internal control, cells co-expressing p35, an inhibitor of caspases, had 14.7% apoptosis (Figure 5A). Thus, Akt inhibits MEKK1-induced apoptosis.

Akt blocks apoptosis by multiple mechanisms, including activation anti-apoptotic proteins and by inhibition of pro-apoptotic proteins. To determine if Akt specifically blocks MEKK1-induced caspase activation, HEK 293 cells were transiently transfected with full length MEKK1, with or without the expression of myr-Akt and wt-Akt. Caspase activity was determined using a caspase 3 consensus substrate (DEVE-AFC) which, when cleaved, produced a fluorescent product which was quantified as described in the Materials and Methods section. Expression of MEKK1 alone caused a 2.1 fold increase in caspase 3-like protease activity above baseline. This fold increase was reduced to below basal levels by co-expression with myr-Akt (0.87 fold) and to basal level by co-expression with wt-Akt (1.07 fold) (Figure 5B). These results are consistent with the hypothesis that Akt blocks MEKK1-induced apoptosis by inhibiting caspase 3-like protease activation.

Prevention of MEKK1-induced apoptosis by Akt is not mediated by inhibition of JNK activation

MEKK1 is known to activate c-Jun N-terminal kinase (JNK), a MAP kinase family member. JNK activation has been postulated to be involved with the process of apoptosis induction. To determine if Akt blocks MEKK1-induced apoptosis by blocking activation of the JNK pathway, HEK 293 cells were transiently transfected with both MEKK1 and wt-Akt cDNA, and the level of JNK activation was determined as described in the Material and Methods section. Transfection of MEKK1 alone or with myr-Akt led to equivalent levels of JNK activation (Figure 6) while myr-Akt or vector alone failed to activate JNK activity. Thus, Akt does not block MEKK1-induced JNK activation.

Task 2. To determine the mechanism by which Stat3, an oncogene found to be upregulated in breast cancer tumors, translocates from the plasma membrane to the nucleus.

STATs (Signal Transducers and Activators of Transcription) are transcription factors that regulate genetic programs controlling development, differentiation, proliferation and apoptosis (26,27). Stat3 is a STAT family member that can be activated by diverse cytokines, growth factors or oncoproteins, and has a critical role in cell growth and survival (28). Upon stimulation of cell surface receptors, Stat3 protein is recruited to activated receptors through an interaction between the Stat3 SH2 domain and phosphotyrosine docking sites on the

receptors (26,27). Subsequent tyrosine phosphorylation of Stat3 occurs directly via the receptor kinase itself or indirectly by activation of intermediary kinases, including members of the Janus kinase (JAK) and Src families, thereby inducing Stat3 dimerization (28). Following dimerization, Stat3 translocates to the nucleus and binds to specific DNA sequences in the promoters of genes and induces their expression through interactions with other transcriptional regulatory components (27).

The mode by which STAT family proteins translocate to the nucleus following their activation in the cytoplasm has remained unclear. Evidence for an active role of endocytosis in signal transduction pathways has been accumulating (29-31). For example, positive signaling during growth factor receptor endocytosis occurs following association of EGF receptor, mSos, Ras, and Raf-1 on endosomes (32). Furthermore, activation of phosphatidylinositol 3-kinase and extracellular signal-regulated kinase (Erk) is attenuated when receptor endocytosis is inhibited (30,33,34). To determine the mechanism by which Stat3 translocates through the cytoplasm to the nucleus, we investigated the hypothesis that Stat3 is actively targeted to the nucleus by transport on vesicles derived from endocytosis.

Receptor-mediated endocytosis allows the specific removal of cell surface receptors and their cargo from the plasma membrane and targets them to endosomes, where they are sorted for downregulation or recycling (5, 10). This process is initiated upon ligand binding by recruitment of the receptor complex into a clathrin-coated pit at the plasma membrane, a structure formed by assembly of clathrin and clathrin adaptor protein 2 (AP-2) into a protein lattice on the membrane's cytosolic face (35). Clathrin and AP-2 bind to multiple components of the endocytic complex such as amphiphysin, Eps15, and epsin (36). These proteins in turn bind to additional proteins that modulate formation and function of clathrin-coated pits, including dynamin, intersectin, and synaptojanin (36). Amphiphysin 1 interacts with dynamin and synaptojanin via its carboxyl-terminal SH3 domain, whereas its central region binds to AP-2 and clathrin (37-40). Expression of the AP-2 and clathrin-binding fragment of amphiphysin 1 (Amph A1) leads to mislocalization of AP-2 and clathrin, with a resultant block in clathrin-mediated endocytosis (39). Another protein important for receptor endocytosis, epsin, binds to clathrin, AP-2, Eps15, and intersectin by multiple domains (41,42). Overexpression of full-length Epsin 2a mislocalizes endocytic complexes and inhibits clathrin-mediated endocytosis (41,43). Using various specific inhibitors of endocytosis, we examined the role of receptor-mediated endocytosis in Stat3 activation and function.

Stat3 translocation into the nucleus following growth factor stimulation requires functional endocytosis. Previous studies showed that endocytosis of epidermal growth factor receptor (EGF-R) is blocked by ectopic expression of an Amph A1 fragment or Epsin 2a protein (39,41). NIH-3T3 cells overexpressing EGF-R (NIH-3T3/EGF-R cells) were transfected with expression vectors encoding either of these endocytic inhibitor proteins or control empty vector and then stimulated with EGF following serum starvation for 12 h. In some experiments, 5 μM phenylarsine oxide (PAO), a pharmacologic inhibitor of EGF-R endocytosis (44,45), was added for the last one-half hour of the serum-free treatment and included in the media during the time of growth factor addition. Nuclear extracts were prepared and incubated with a ^{32}P-labeled high-affinity sis-inducible element (hSIE) oligonucleotide probe (46), and the resultant DNA-protein complexes were analyzed by electrophoretic mobility shift assay (EMSA) for activated Stat3 dimers. Nuclear DNA-binding activity of endogenous Stat3 in response to EGF treatment was greatly inhibited by overexpression of Amph A1 or Epsin 2a (Fig. 7A). Further, blocking endocytosis of EGF-R with PAO resulted in a complete loss of Stat3 DNA-binding activity in response to EGF. Analysis of cytoplasmic Stat3 in response to EGF show a block in dimer formation and DNA-binding activity following inhibition of endocytosis (data not shown). Functional endocytosis was also required for Stat3 nuclear DNA-binding activity following platelet-derived growth factor (PDGF) treatment of Balb/c-3T3 cells expressing endogenous levels of PDGF-R (Fig. 7C). These results demonstrate that translocation of active Stat3 to the nucleus in response to growth factor stimulation is dependent on the endocytic pathway.

Stat3 phosphorylation is independent of nuclear translocation and receptor endocytosis.

EGF-R autophosphorylation, Ras and Raf-1 activation, and phosphorylation of Shc following growth factor stimulation are not inhibited when endocytosis is blocked (44,47,48). Therefore, while growth factor receptor endocytosis is blocked by expression of the endocytic inhibitors, it is possible that ligand-induced Stat3 phosphorylation is not inhibited. To further define the mechanism by which blocking endocytosis inhibits Stat3 DNA-binding activity, we examined tyrosine phosphorylation of Stat3 following EGF stimulation. NIH-3T3/EGF-R cells expressing control empty vector, Amph A1, or Epsin 2a, or treated with PAO (as described above) were lysed following EGF stimulation, and Stat3 was immunoprecipitated from lysates. Western blotting was performed using an anti-phosphoY^{705}-Stat3 antibody, which is specific for activated Stat3. Tyrosine phosphorylation of Stat3 in response to EGF was not affected by inhibition of endocytosis (Fig. 7B). Additionally, inhibition of endocytosis does not block Stat3 tyrosine phosphorylation following PDGF stimulation of Balb/c-3T3 cells (Fig. 7D). These results indicate that growth factors bind to and activate their receptors in the presence of endocytic inhibitors, thereby allowing Stat3 phosphorylation. Thus, Amph A1, Epsin 2a, and PAO inhibit events subsequent to Stat3 phosphorylation, suggesting a requirement for receptor endocytosis in Stat3 nuclear translocation and that Stat3 tyrosine phosphorylation alone is not sufficient for nuclear translocation.

Stat3 transcriptional activity requires receptor-mediated endocytosis.

Because nuclear Stat3 DNA-binding activity was inhibited following a block in endocytosis, we further investigated the role of endocytosis in Stat3-mediated gene regulation induced by growth factor stimulation. Balb/c-3T3 cells were transfected with empty vector or vectors encoding endocytic inhibitors and the Stat3-responsive reporter plasmid, pLucTKS3, which contains multiple copies of a Stat3-specific DNA-binding site driving luciferase expression (49). As shown in Fig. 7E, Stat3-mediated transcriptional activity in response to PDGF was abrogated by expression of the endocytic inhibitors, demonstrating that blocking endocytosis prevents nuclear translocation of functional Stat3 dimers. Collectively, these results indicate a requirement of receptor-mediated endocytosis for induction of Stat3 DNA-binding activity and transcriptional activity in the nucleus.

Stat3 translocates from the cell membrane to the perinuclear region in endosomes following growth factor treatment.

To further illustrate the contribution of growth factor receptor endocytosis in Stat3 signaling, confocal microscopy immunofluorescence studies with antibodies to either Stat3 or AP-2, a marker for endocytic vesicles, were used to examine co-localization of endogenous Stat3 with endosomes following PDGF stimulation (Fig. 8A-C). NIH-3T3 cells were treated with PDGF for 45 min at 4°C, which allows localization of PDGF-bound receptor to the plasma membrane but is a non-permissive temperature for endocytosis. Cells were then shifted to 37 °C for the times indicated. Endogenous Stat3 localized with AP-2 at the plasma membrane following a 4°C incubation with PDGF (Fig. 8A), consistent with targeting of Stat3 to forming clathrin-coated vesicles upon growth factor binding to its receptor. After a 10 min treatment with growth factor at 37°C, Stat3 co-localized with AP-2 in the cytosol, indicating that endogenous Stat3 is internalized with endocytic vesicles (Fig. 8B). Subsequently, endogenous Stat3 localized to endocytic vesicles in the perinuclear region after 30 min treatment with PDGF at 37°C (Fig. 8C). Stat3 similarly co-localizes with AP-2 following EGF treatment of NIH-3T3 cells (data not shown). Also, to use an independent method of Stat3 detection, we expressed a Stat3-red fluorescent protein (Stat3-RFP) chimera in NIH-3T3 cells. Following EGF treatment, Stat3-RFP co-localized with AP-2 in endocytic vesicles in transit from the plasma membrane to the nucleus (data not shown). Together, these findings demonstrate that Stat3 localizes to endocytic vesicles that shuttle from the plasma membrane to the perinuclear region following growth factor stimulation.

Growth factor receptor-ligand complexes scaffold Stat3 from the plasma membrane to the perinuclear region during endocytosis.

Activation of PDGF and EGF receptors leads to Stat3 activation, and previous studies showed direct Stat3 interaction with PDGF and EGF receptors (50,51). To further examine the role of receptors in this translocation, a fluorescent EGF conjugate (Alexa Fluor EGF, Molecular Probes) was used to detect the localization of EGF

receptor-ligand complexes. For these confocal microscopy experiments, Stat3 was overexpressed in order to achieve levels of brightness equivalent to the fluorescent EGF for co-localization studies. Following transfection with Stat3 expression vector and treatment with Alexa Fluor EGF, immunofluorescence studies with antibodies to Stat3 were performed to determine co-localization with EGF receptor-ligand complexes. As shown in Fig. 9A-C, Stat3 co-localizes with the EGF receptor-ligand complexes following treatment with Alexa Fluor EGF in endosomal compartments sequentially at the cell membrane at 0 time (panel A), in the cytoplasm at 10 min (panel B), and the perinuclear region at 30 min (panel C). Elevated Stat3 nuclear levels, which are detectable in the absence of EGF stimulation due to Stat3 overexpression, increase significantly during the 30 min time course following EGF stimulation. Similar results were obtained following co-transfection of expression vectors encoding EGF-R fused to green-fluorescence protein (GFP) and Stat3-RFP (data not shown). These results suggest that growth factor receptors serve as a scaffold for Stat3 trafficking from the plasma membrane to the perinuclear region during endocytosis.

Inhibition of functional receptor endocytosis blocks Stat3 trafficking from the cell membrane to the perinuclear region.

The above experiments show that Stat3 localizes with endocytic vesicles and EGF receptor-ligand complexes following growth factor stimulation. To further characterize the requirement of endocytosis for Stat3 translocation, cells overexpressing Stat3 and either Amph A1 or Epsin 2a were treated with EGF, and confocal microscopy immunofluorescence studies were used to determine the localization of Stat3. As shown in Fig. 10A-C, Stat3 transport through the cytoplasm following EGF treatment was blocked by overexpression of either Amph A1 or Epsin 2a or treatment with PAO at times 0 (panel A), 10 min (panel B) and 30 min (panel C). No significant accumulation of Stat3 in the nucleus was detected. Therefore, Stat3 translocation to the perinuclear region in response to EGF stimulation requires functional endocytosis.

Functional receptor-mediated endocytosis is necessary for both IL-6 and v-Src mediated Stat3 activation and transcriptional regulation.

In addition to growth factor activation, Stat3 can be activated by IL-6 and Src stimulation (46,52,53). Interleukin-6 (IL-6) exerts its action via a receptor complex consisting of two subunits, the IL-6 receptor and the signal transducer gp130, which undergoes endocytosis (54). To determine whether endocytosis is a general mechanism required for Stat3 activation, we examined Stat3 activation following IL-6 stimulation or in cells expressing constitutively activation v-Src with or without 5 μM PAO treatment. EMSA for activated Stat3 dimers was analyzed as described. Cytoplasmic and nuclear DNA-binding activity of endogenous Stat3 in response to IL-6 treatment or v-Src activation was inhibited by blocking endocytosis (Fig. 11A-B). Also, v-Src mediated Stat3 transcriptional activation requires functional endocytosis (Fig. 11C). These results demonstrate that translocation of active Stat3 to the nucleus in response to IL-6 and v-Src stimulation is dependent on the endocytic pathway.

KEY RESEARCH ACCOMPLISHMENTS:

The mechanism by which MEKK1 causes apoptosis, and the ability of the Akt oncogene to subvert this apoptotic pathway has been examined:

- MEKK1-induced apoptosis requires death receptor activation.
- Furthermore, expression of 91 kDa MEKK1 induces increased DR4, Fas and caspase-8 mRNA and protein levels.
- MEKK1-induced apoptosis is potentiated by blocking PI-3 kinase activation.
- Akt, a serine-threonine kinase downstream of PI-3 kinase, prevents the cleavage of endogenous MEKK1 by genotoxins when overexpressed.
- Cells with homozygous deletion of caspase 3 do not produce MEKK1 cleavage in response to etoposide treatment. Further, Akt blocks MEKK1-induced apoptosis and caspase 3-like activation.
- Akt did not block MEKK1-induced JNK activation, showing that regulation of the JNK pathway by MEKK1 is independent of its role in regulation of apoptosis.

Thus, MEKK1-induced apoptosis requires cleavage by caspase 3 and activation of the TRAIL death receptor pathway. MEKK1-induced death is potentiated by inhibition of PI-3 kinase, and is blocked by Akt through inhibition of MEKK1 cleavage.

The mechanism by which Stat3 translocates from the plasma membrane to the nucleus has been determined:

- Stat3 cytoplasmic and nuclear DNA-binding activity requires functional endocytosis.
- Stat3 transcriptional activity requires endocytosis.
- Stat3 tyrosine phosphorylation is not dependent on endocytosis.
- The mechanism whereby one activated STAT family member, Stat3, is transported through the cytoplasm involves receptor-mediated endocytosis.
- Following growth factor stimulation, Stat3 co-localizes with receptors in endocytic vesicles that are in transit to the perinuclear region.
- Disruption of endocytosis with specific inhibitors blocks Stat3 nuclear translocation and Stat3-dependent gene regulation.
- Inhibition of endocytosis also blocks IL-6 and v-Src induced Stat3 activation.

Therefore, receptor-mediated endocytosis may be a general mechanism for transport of cytoplasmic signaling proteins to the nucleus.

REPORTALE OUTCOMES:

Manuscripts:

Andrea H. Bild, James Turkson and Richard Jove. Cytoplasmic Transport of Stat3 by Receptor-mediated Endocytosis. *Submitted to Science Sept. 29, 2001.*

Andrea H. Bild, Erika M. Gibson, Mei Huang, Jacylyn Onio, Timothy P. Garrington, Richard Jove, Gary L. Johnson, and Spencer B. Gibson. MEKK1-induced apoptosis requires TRAIL death receptor activation and is inhibited by Akt/PKB through inhibition of MEKK1 cleavage. *In Preperation.*

Tatiana Sorkina, Andrea Bild, Francesc Tebar, and Alexander Sorkin. Clathrin, adaptors and eps15 in endosomes containing activated epidermal growth factor receptors. Journal of Cell Science. 112, 317-327 (1999).

Abstracts:

MEKK1 and Akt: Opponents in Cell Death. Andrea H. Bild, Gary L. Johnson and Spencer B. Gibson. Department of Defense, Era of Hope Meeting, Atlanta, GA, June 8-12, 2000.

Presentations:

Mechanism of MEKK1 and Stat3 Regulation of Signaling Pathways. Andrea H. Bild, Gary L. Johnson and Richard Jove. University of Colorado Health Science Center, Denver, CO, June, 2001.

CONCLUSIONS:

Task 1:

Together, our results clearly demonstrate that death receptor activation is required for MEKK1-mediated apoptosis. This mechanism involves upregulation of DR4 and Fas death receptor levels. Inhibition of upstream regulators of Akt potentiates MEKK1-induced apoptosis. Akt is able to block MEKK1-induced apoptosis and caspase amplification by inhibiting cleavage of MEKK1 by caspase 3, thus preventing release of the pro-apoptotic 91 kDa kinase fragment of MEKK1. MEKK1 activation also increases Fas ligand expression in Jurkat T cells, suggesting that MEKK1 regulation of death receptor activation is an important mechanism for induction of apoptosis in different cell types (55,56).

It is becoming more important to characterize the relationship between apoptotic and oncogenic signaling pathways in order to tailor more targeted therapies for cancer cells. Knowledge of mechanisms by which cells regulate apoptosis can lead to potentiation of these pathways using specialized chemotherapies. Further, delineation of oncogenic pathways that subvert apoptotic pathways or effects of chemotherapeutic reagents will allow generation of more targeted cancer treatments to block these effects.

Activation of Akt has been shown to block caspase activation and apoptosis following treatment with many different apoptotic stimuli, including anoikis and genotoxic agents and therefore potentially contributes to chemotherapy resistance in some forms of cancer. Interestingly, MEKK1 is necessary for the induction of apoptosis following anoikis and specific genotoxic treatments (6,57). Akt prevents MEKK1-induced apoptosis and caspase activation. Akt also prevents MEKK1 cleavage. However, Akt fails to block etoposide-mediated MEKK1 kinase activity and upregulation of DR4 and DR5 expression (data not shown). Further, Akt inhibition of etoposide-induced apoptosis is not through inhibition of DR4 and DR5 activation or caspase 8 activation (results not shown). The direct mechanism by which Akt blocks MEKK1 induced caspase activation is unknown, but our findings indicate that it is through inhibition of caspase 3 activity and subsequent MEKK1 cleavage to its 91 kDa domain and further caspase amplification.

Evidence suggests that death receptor activation plays a role in genotoxin-induced apoptosis. For example, increased expression of Fas ligand following doxorubicin treatment contributes to the induction of apoptosis (58). Also, treatment with etoposide results in increased expression of DR4 and DR5 and is a proposed mechanism for genotoxin-induced apoptosis (7). The ligand for DR4 and DR5, TRAIL, is a promising new molecular-based drug for treatment of cancer. Inhibition of the binding of TRAIL ligand to DR4 and DR5 reduces the apoptotic response to etoposide (7). Further, preliminary studies have shown its ability to reduce the size of human tumors in mice. In combination with genotoxic agents, TRAIL can eradicate some human tumors in mice and can lead to a synergistic apoptotic response in some breast cancer cell lines (7,59,60). These responses could at least partially be explained by the up-regulation of DR4 and DR5 expression. We have shown that Akt is effective at blocking MEKK1 and etoposide-induced apoptosis but not DR4 and DR5 upregulation and activation. It follows that Akt will most likely not inhibit the early synergistic apoptotic response of TRAIL and genotoxin treatment, but instead will affect downstream apoptotic responses, including MEKK1 cleavage and amplification of caspase activation. Alternatively, blocking Akt or its upstream regulators could potentially result in enhancement of the apoptotic signal by TNF death receptor pathways, particularly cells with increased expression of Akt.

Activation of MEKK1 leads to JNK activation. JNK has been implicated in the induction of apoptosis. In the presence of Akt, MEKK1 mediated JNK activation was not affected. This result suggests that JNK activation is not responsible for MEKK1's pro-apoptotic effects and that Akt's prevention of MEKK1-induced apoptosis is unrelated to MEKK1's regulation of JNK. It is still possible, however, that Akt could block downstream events following JNK activation leading to inhibition of apoptosis. Since expression of kinase inactive MEKK1 fails to block JNK activation by etoposide (data not presented) but effectively blocks etoposide-induced apoptosis, it is unlikely that JNK is playing a major role in MEKK1-induced apoptosis. Indeed, MEKK1 knock out fibroblasts

show a decreased JNK response to cell stresses that alter the cytoskeletal structure and an increased apoptotic response to these stresses, suggesting that MEKK1-induced JNK activation may actually be protective (61).

Cumulatively, our results show that death receptor activation plays an important role in MEKK1-induced apoptosis and that Akt blocks MEKK1-induced apoptosis and caspase activation. This anti-apoptotic effect involves the inhibition of MEKK1 cleavage, downstream of death receptor activation. These findings thus identify potential molecular targets that may be effective at regulating TNF receptor and MEKK1-mediated apoptosis and overcoming survival signals, leading to more targeted cancer therapies.

Task 2:

Our data support a model in which growth factors and cytokine receptors serve as a scaffold for Stat3 transport by endocytic vesicles from the plasma membrane to the perinuclear region. Evidence suggests that EGF is released from its receptor in late endosomes before degradation (62), which may provide a mechanism by which Stat3 is released from the receptor-ligand complex and is subsequently imported into the nucleus. Alternatively, recent work suggests that EGF-R functions as a transcription factor and is localized to the nucleus in highly proliferating tissues (63). Therefore, EGF-R may shuttle Stat3 directly into the nucleus. Further, certain components of the endocytic machinery, including Eps15, Eps15R and AP-180, possess FG repeats (39,41,43). FG-containing repeat regions have been shown to interact with several members of the importin family (64). It is possible, therefore, that endocytic proteins interact with members of the importin family, connecting the molecular machineries governing vesicle sorting and nucleo-cytoplasmic shuttling. Furthermore, Epsin 1 undergoes nucleo-cytoplasmic shuttling and its ENTH domain is structurally similar to Armadillo and HEAT repeats of β-catenin and karyopherin-β, respectively (65), and thus potentially participates in nuclear transport directly. However, the precise mechanism of how Stat3 translocates across the nuclear membrane remains to be determined.

There is literature to support a role of Src in endocytic trafficking of receptor tyrosine kinases by binding and activating dynamin (66) and via phosphorylation, causing clathrin redistribution to the cell periphery (67). Furthermore, a significant fraction of c-Src in fibroblasts has been found associated with endosomes (68,69). Overexpression of Src leads to an increase in the rate of endocytosis (70), and experiments in cells lacking Src family members or cells treated with the Src inhibitor (PPI) show a delay in activated receptor endocytosis (67). Our results show that blocking endocytosis inhibits Src-induced Stat3 nuclear DNA-binding activity and transcriptional activation, suggesting that non-receptor tyrosine kinase Stat3 activation similarly requires endocytosis. Further, Src may contribute to Stat3 activation by enabling or enhancing endocytosis.

In summary, our results demonstrate that endocytosis is necessary for Stat3 translocation from the cytoplasm to the nucleus. Stat3 tyrosine phosphorylation is not sufficient for full activation of Stat3 function. Furthermore, endocytosis of growth factor or cytokine receptors serves to transport Stat3 from the plasma membrane to the perinuclear region following ligand stimulation. Together, these results indicate for the first time a mechanism by which Stat3 is actively transported through the cytoplasm to the nucleus via endocytic vesicles. These findings also illustrate a novel role of growth factor receptors as scaffolds in cell membrane-perinuclear transit. This mechanism may also apply to unrelated signaling proteins such as Erk and Smad that undergo nuclear translocation following cytoplasmic activation.

REFERENCES:

1. Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J., and Johnson, G. L. (1993) *Science* **260**(5106), 315-9.
2. Widmann, C., Gibson, S., Jarpe, M. B., and Johnson, G. L. (1999) *Physiol Rev* **79**(1), 143-80.
3. Widmann, C., Gerwins, P., Johnson, N. L., Jarpe, M. B., and Johnson, G. L. (1998) *Mol Cell Biol* **18**(4), 2416-29.
4. Schlesinger, T. K., Fanger, G. R., Yujiri, T., and Johnson, G. L. (1998) *Front Biosci* **3**(5395), D1181-6.
5. Deak, J. C., Cross, J. V., Lewis, M., Qian, Y., Parrott, L. A., Distelhorst, C. W., and Templeton, D. J. (1998) *Proc Natl Acad Sci U S A* **95**(10), 5595-600.
6. Cardone, M. H., Salvesen, G. S., Widmann, C., Johnson, G., and Frisch, S. M. (1997) *Cell* **90**(2), 315-23.
7. Gibson, S. B., Oyer, R., Spalding, A. C., Anderson, S. M., and Johnson, G. L. (2000) *Mol Cell Biol* **20**(1), 205-12.
8. Soh, J. W., Mao, Y., Liu, L., Thompson, W. J., Pamukcu, R., and Weinstein, I. B. (2001) *J Biol Chem* **276**(19), 16406-10.
9. Gebauer, G., Mirakhur, B., Nguyen, Q., Shore, S. K., Simpkins, H., and Dhanasekaran, N. (2000) *Int J Oncol* **16**(2), 321-5.
10. Ashkenazi, A., and Dixit, V. M. (1999) *Curr Opin Cell Biol* **11**(2), 255-60.
11. Golstein, P. (1997) *Curr Biol* 7(12), R750-3.
12. Green, D. R. (2000) *Cell* **102**(1), 1-4.
13. Lee, S. H., Shin, M. S., Kim, H. S., Lee, H. K., Park, W. S., Kim, S. Y., Lee, J. H., Han, S. Y., Park, J. Y., Oh, R. R., Kang, C. S., Kim, K. M., Jang, J. J., Nam, S. W., Lee, J. Y., and Yoo, N. J. (2001) *Oncogene* **20**(3), 399-403.
14. Shin, M. S., Kim, H. S., Lee, S. H., Park, W. S., Kim, S. Y., Park, J. Y., Lee, J. H., Lee, S. K., Lee, S. N., Jung, S. S., Han, J. Y., Kim, H., Lee, J. Y., and Yoo, N. J. (2001) *Cancer Res* **61**(13), 4942-6.
15. Mullauer, L., Gruber, P., Sebinger, D., Buch, J., Wohlfart, S., and Chott, A. (2001) *Mutat Res* **488**(3), 211-31.
16. Straus, S. E., Jaffe, E. S., Puck, J. M., Dale, J. K., Elkon, K. B., Rosen-Wolff, A., Peters, A. M., Sneller, M. C., Hallahan, C. W., Wang, J., Fischer, R. E., Jackson, C. M., Lin, A. Y., Baumler, C., Siegert, E., Marx, A., Vaishnaw, A. K., Grodzicky, T., Fleisher, T. A., and Lenardo, M. J. (2001) *Blood* **98**(1), 194-200.
17. Sheridan, J. P., Marsters, S. A., Pitti, R. M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C. L., Baker, K., Wood, W. I., Goddard, A. D., Godowski, P., and Ashkenazi, A. (1997) *Science* **277**(5327), 818-21.
18. Sun, M., Wang, G., Paciga, J. E., Feldman, R. I., Yuan, Z. Q., Ma, X. L., Shelley, S. A., Jove, R., Tsichlis, P. N., Nicosia, S. V., and Cheng, J. Q. (2001) *Am J Pathol* **159**(2), 431-7.
19. Blume-Jensen, P., and Hunter, T. (2001) *Nature* **411**(6835), 355-65.
20. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) *Genes Dev* **13**(22), 2905-27.
21. Khwaja, A. (1999) *Nature* **401**(6748), 33-4.
22. Di Cristofano, A., Kotsi, P., Peng, Y. F., Cordon-Cardo, C., Elkon, K. B., and Pandolfi, P. P. (1999) *Science* **285**(5436), 2122-5.
23. Panka, D. J., Mano, T., Suhara, T., Walsh, K., and Mier, J. W. (2001) *J Biol Chem* **276**(10), 6893-6.
24. Tschopp, J., Irmler, M., and Thome, M. (1998) *Curr Opin Immunol* **10**(5), 552-8.
25. Pan, G., Ni, J., Wei, Y. F., Yu, G., Gentz, R., and Dixit, V. M. (1997) *Science* **277**(5327), 815-8.
26. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) *Science* **264**(5164), 1415-21.
27. Darnell, J. E., Jr. (1997) *Science* **277**(5332), 1630-5.
28. Bowman, T., Garcia, R., Turkson, J., and Jove, R. (2000) *Oncogene* **19**(21), 2474-88.
29. McPherson PS, K. B., Hussain NK. (2001) *Traffic* **2**, 375-384
30. Ceresa, B. P., and Schmid, S. L. (2000) *Curr Opin Cell Biol* **12**(2), 204-10.
31. Leof, E. B. (2000) *Trends Cell Biol* **10**(8), 343-8.

32. Di Guglielmo, G. M., Baass, P. C., Ou, W. J., Posner, B. I., and Bergeron, J. J. (1994) *Embo J* **13**(18), 4269-77.
33. Ceresa, B. P., Kao, A. W., Santeler, S. R., and Pessin, J. E. (1998) *Mol Cell Biol* **18**(7), 3862-70.
34. Vieira, A. V., Lamaze, C., and Schmid, S. L. (1996) *Science* **274**(5295), 2086-9.
35. Robinson, M. S., Watts, C., and Zerial, M. (1996) *Cell* **84**(1), 13-21.
36. Di Fiore, P. P., and Gill, G. N. (1999) *Curr Opin Cell Biol* **11**(4), 483-8.
37. Bauerfeind, R., Takei, K., and De Camilli, P. (1997) *J Biol Chem* **272**(49), 30984-92.
38. Ramjaun, A. R., Philie, J., de Heuvel, E., and McPherson, P. S. (1999) *J Biol Chem* **274**(28), 19785-91.
39. Slepnev, V. I., Ochoa, G. C., Butler, M. H., and De Camilli, P. (2000) *J Biol Chem* **275**(23), 17583-9.
40. Takei, K., Slepnev, V. I., Haucke, V., and De Camilli, P. (1999) *Nat Cell Biol* **1**(1), 33-9.
41. Chen, H., Fre, S., Slepnev, V. I., Capua, M. R., Takei, K., Butler, M. H., Di Fiore, P. P., and De Camilli, P. (1998) *Nature* **394**(6695), 793-7.
42. Rosenthal, J. A., Chen, H., Slepnev, V. I., Pellegrini, L., Salcini, A. E., Di Fiore, P. P., and De Camilli, P. (1999) *J Biol Chem* **274**(48), 33959-65.
43. Chen, H., Slepnev, V. I., Di Fiore, P. P., and De Camilli, P. (1999) *J Biol Chem* **274**(6), 3257-60.
44. Tong, X. K., Hussain, N. K., Adams, A. G., O'Bryan, J. P., and McPherson, P. S. (2000) *J Biol Chem* **275**(38), 29894-9.
45. Hertel, C., Coulter, S. J., and Perkins, J. P. (1985) *J Biol Chem* **260**(23), 12547-53.
46. Yu, C. L., Meyer, D. J., Campbell, G. S., Larner, A. C., Carter-Su, C., Schwartz, J., and Jove, R. (1995) *Science* **269**(5220), 81-3.
47. Johannessen, L. E., Ringerike, T., Molnes, J., and Madshus, I. H. (2000) *Exp Cell Res* **260**(1), 136-45.
48. Kao, A. W., Ceresa, B. P., Santeler, S. R., and Pessin, J. E. (1998) *J Biol Chem* **273**(39), 25450-7.
49. Turkson, J., Bowman, T., Garcia, R., Caldenhoven, E., De Groot, R. P., and Jove, R. (1998) *Mol Cell Biol* **18**(5), 2545-52.
50. Wang, Y. Z., Wharton, W., Garcia, R., Kraker, A., Jove, R., and Pledger, W. J. (2000) *Oncogene* **19**(17), 2075-85.
51. Fu, X. Y., and Zhang, J. J. (1993) *Cell* **74**(6), 1135-45.
52. Bromberg, J. F., Horvath, C. M., Besser, D., Lathem, W. W., and Darnell, J. E., Jr. (1998) *Mol Cell Biol* **18**(5), 2553-8.
53. Zhong, Z., Wen, Z., and Darnell, J. E., Jr. (1994) *Science* **264**(5155), 95-8.
54. Dittrich, E., Rose-John, S., Gerhartz, C., Mullberg, J., Stoyan, T., Yasukawa, K., Heinrich, P. C., and Graeve, L. (1994) *J Biol Chem* **269**(29), 19014-20.
55. Faris, M., Latinis, K. M., Kempiak, S. J., Koretzky, G. A., and Nel, A. (1998) *Mol Cell Biol* **18**(9), 5414-24.
56. Faris, M., Kokot, N., Latinis, K., Kasibhatla, S., Green, D. R., Koretzky, G. A., and Nel, A. (1998) *J Immunol* **160**(1), 134-44.
57. Gibson, S., Widmann, C., and Johnson, G. L. (1999) *J Biol Chem* **274**(16), 10916-22.
58. Friesen, C., Herr, I., Krammer, P. H., and Debatin, K. M. (1996) *Nat Med* **2**(5), 574-7.
59. Griffith, T. S., and Lynch, D. H. (1998) *Curr Opin Immunol* **10**(5), 559-63.
60. Gura, T. (1997) *Science* **277**(5327), 768.
61. Yujiri, T., Sather, S., Fanger, G. R., and Johnson, G. L. (1998) *Science* **282**(5395), 1911-4.
62. Burke, P., Schooler, K., and Wiley, H. S. (2001) *Mol Biol Cell* **12**(6), 1897-910.
63. Lin, S. Y., Makino, K., Xia, W., Matin, A., Wen, Y., Kwong, K. Y., Bourguignon, L., and Hung, M. C. (2001) *Nat Cell Biol* **3**(9), 802-8.
64. Ohno, M., Fornerod, M., and Mattaj, I. W. (1998) *Cell* **92**(3), 327-36.
65. Hyman, J., Chen, H., Di Fiore, P. P., De Camilli, P., and Brunger, A. T. (2000) *J Cell Biol* **149**(3), 537-46.
66. Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Hsuan, J., Booker, G. W., and et al. (1993) *Cell* **75**(1), 25-36.
67. Wilde, A., Beattie, E. C., Lem, L., Riethof, D. A., Liu, S. H., Mobley, W. C., Soriano, P., and Brodsky, F. M. (1999) *Cell* **96**(5), 677-87.

68. Kaplan, K. B., Swedlow, J. R., Varmus, H. E., and Morgan, D. O. (1992) *J Cell Biol* **118**(2), 321-33.
69. Redmond, T., Brott, B. K., Jove, R., and Welsh, M. J. (1992) *Cell Growth Differ* **3**(9), 567-76.
70. Ware, M. F., Tice, D. A., Parsons, S. J., and Lauffenburger, D. A. (1997) *J Biol Chem* **272**(48), 30185-90.

Fig. 1. A.

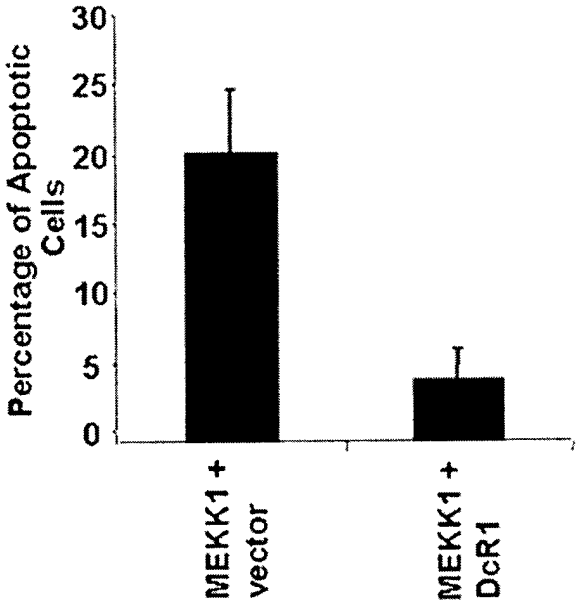

Fig. 1. B.

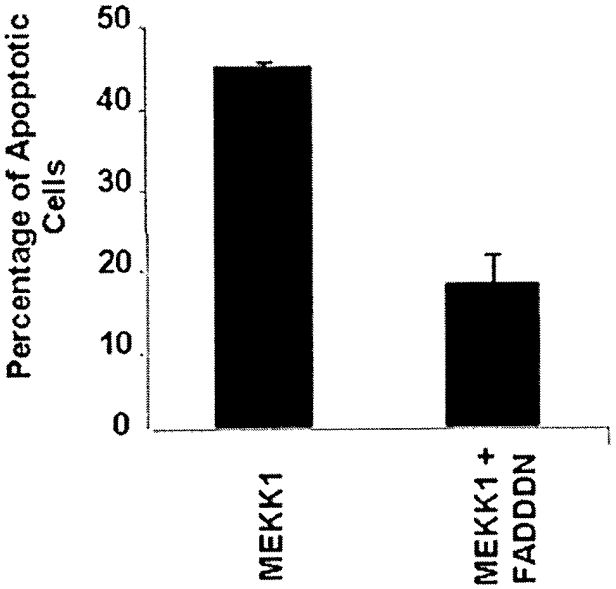

Figure 1 MEKK1-induced apoptosis in HEK293 cells over expressing DcR1 and FADD DN. A) HEK 293 lines stably expressing DcR1 or vector alone were transiently transfected with MEKK1. 48 hours following transfection, the cells were stained for MEKK1 and TdT. The percentage of TdT positive cells expressing MEKK1 determined the percent apoptosis. B) HEK 293 cells were transiently transfected with MEKK1 in the presence or absence of FADD DN. Percent apoptosis was determined by acridine orange staining. Control cells were untransfected or transfected with FADD DN alone. Results are representative of three independent experiments.

Fig. 2A.

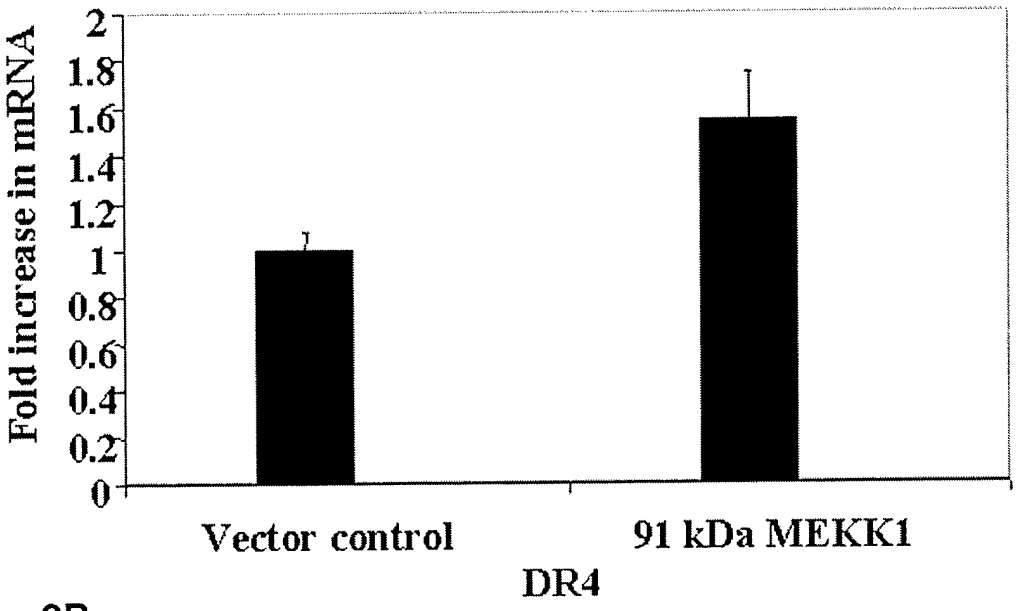

Fig. 2B.

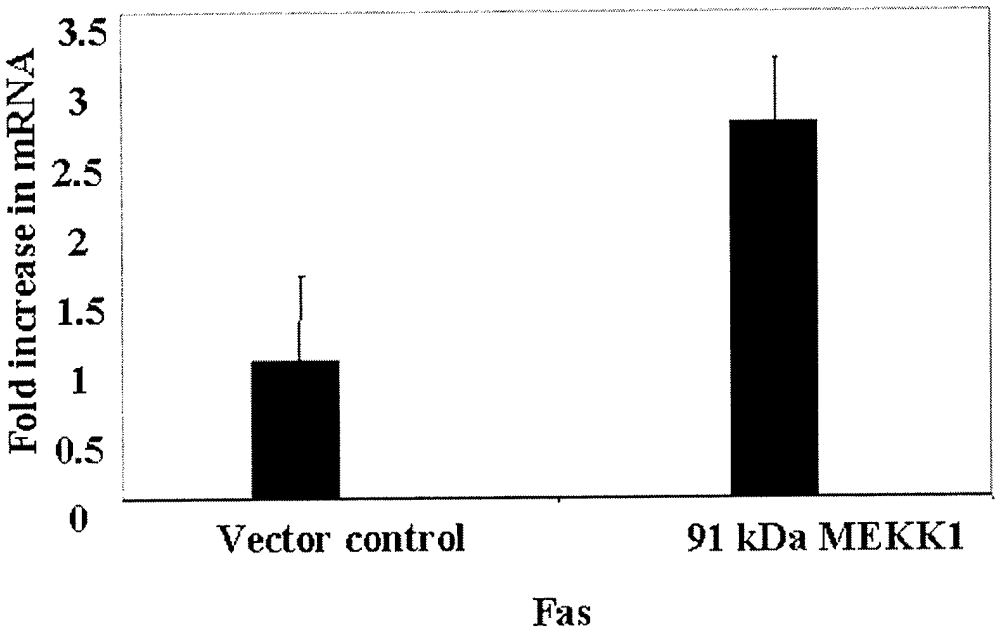

Figure 2 MEKK1 upregulates DR4 and Fas mRNA and protein levels. HEK293 cells were transfected with control vector or a 91kDa MEKK1 vector. 36 h following transfection, RPA analysis was carried out as described in Materials and Methods. Fold increases in A) DR4 and B) Fas were quantitated and standard deviation performed.

Fig. 3

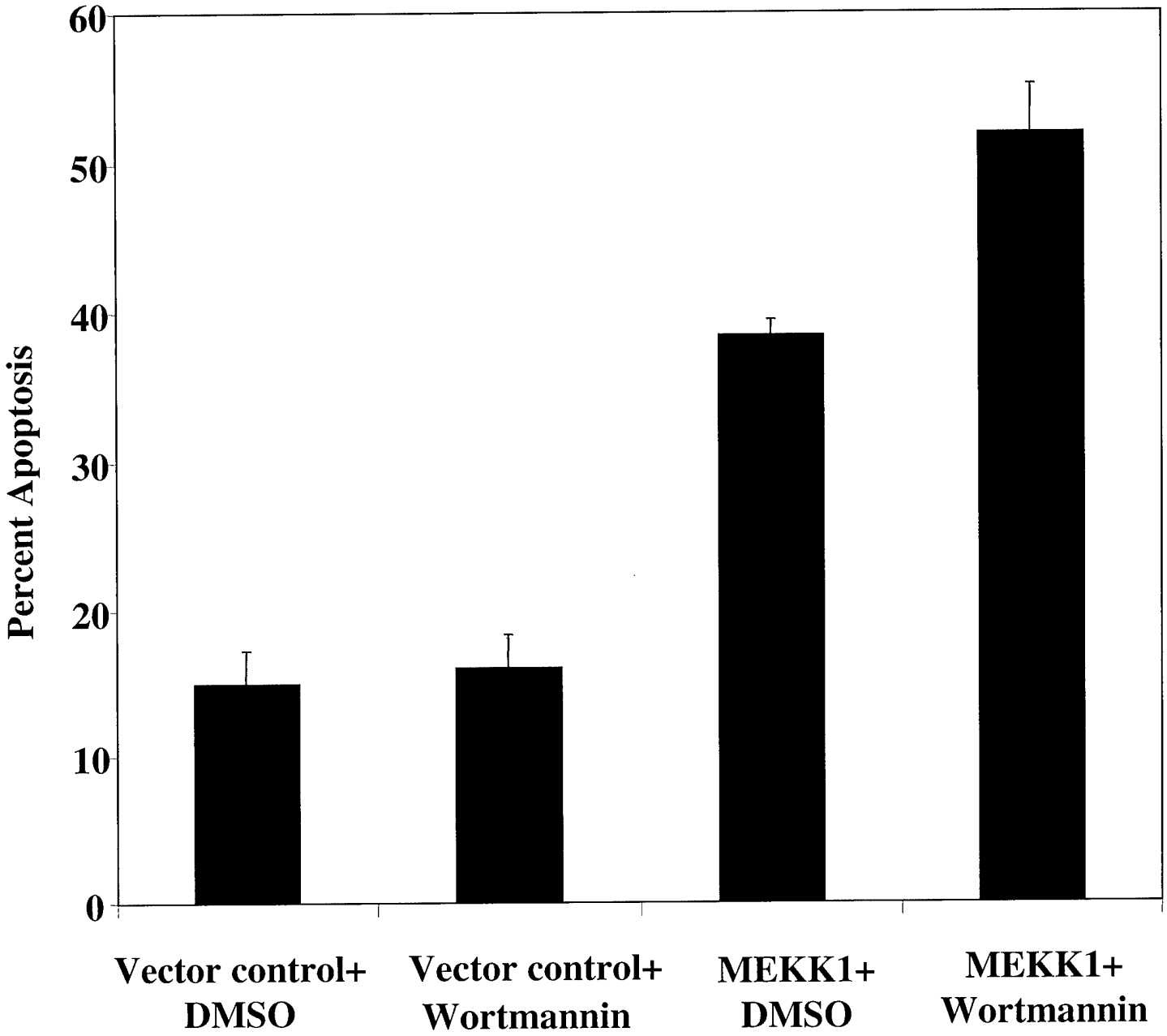

Figure 3 Inhibition of PI-3 kinase potentiates MEKK1-induced apoptosis. HEK293 cells were transfected with a MEKK1-green fluorescent protein (GFP) vector or control GFP vector. 24 h after transfection, cells were serum starved for 12 h with DMSO or 200nm Wortmannin. Cells were then fixed using 3.8% paraformaldehyde and quantified in a blinded manner for expression of GFP protein and apoptotic nuclei.

Fig. 4A

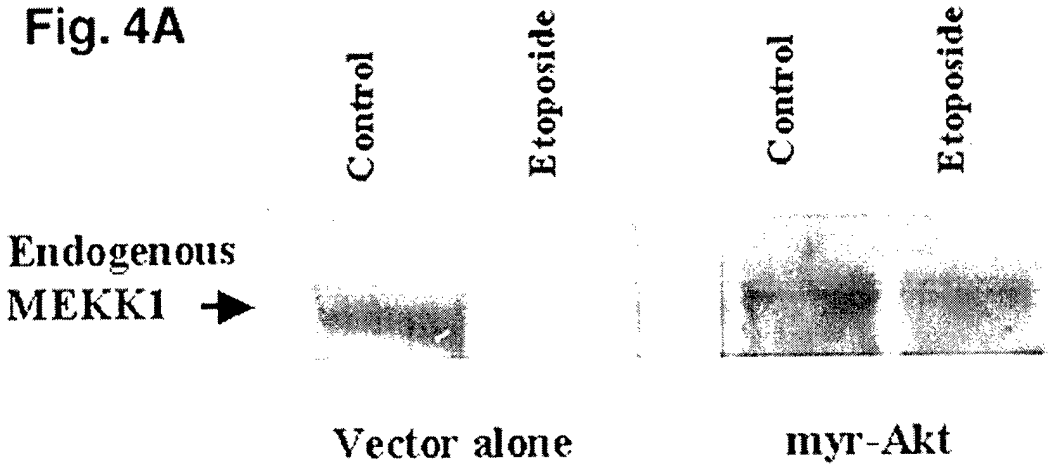

Fig. 4B

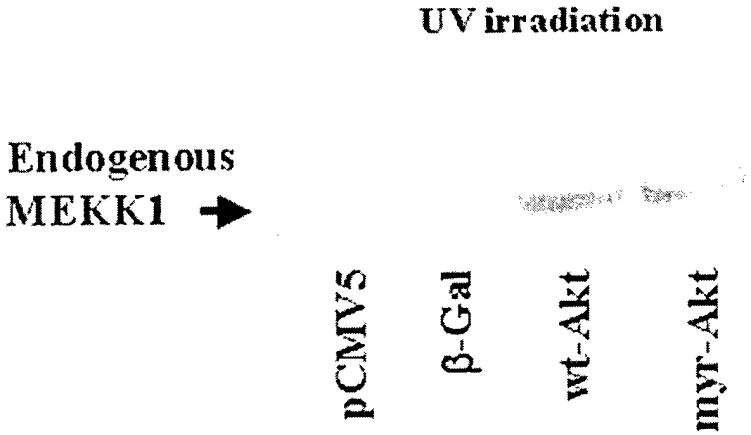

Figure 4 Cleavage of endogenous MEKK1 following etoposide or ultraviolet radiation in HEK 293 cells expressing Akt. HEK 293 cells were treated with 100μM etoposide or ultraviolet irradiation (UV). 48 hours later, the cells were lysed as described in Material and Methods section, and Western blots for endogenous MEKK1 were performed. A) HEK 293 cells stably expressing empty vector or myr-Akt were treated with etoposide for 48 hours and Western blots for MEKK1 were performed. B) HEK 293 cells were transiently transfected with vector alone, wt-Akt, or myr-Akt and treated with 40 J/m^2 UV irradiation. The cells were then lysed, and Western blotted for endogenous MEKK1 were performed.

Fig. 5A

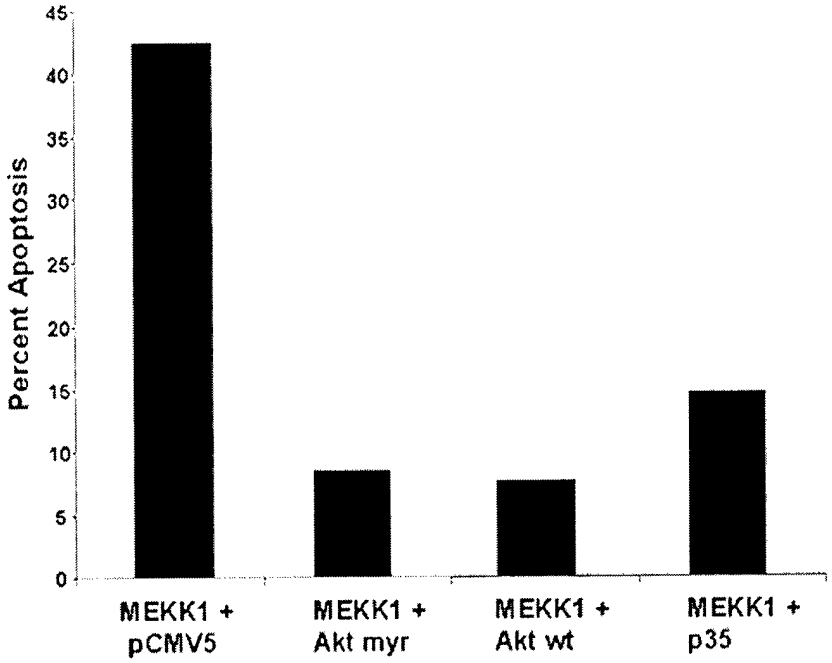

Fig. 5B

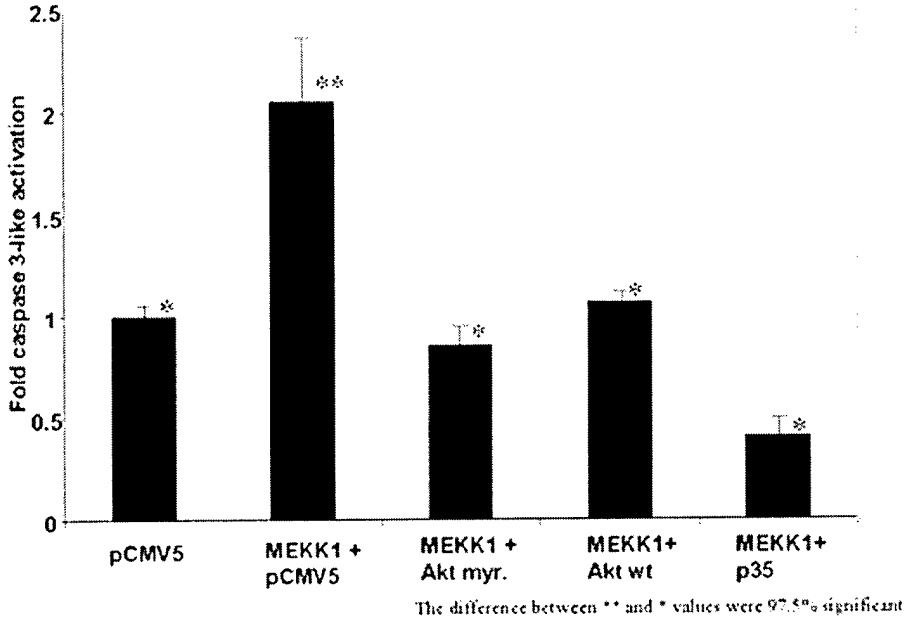

Figure 5 Expression of myr-Akt and wt-Akt inhibit MEKK1-induced apoptosis and caspase activation. A) Cells were analyzed by fluorescence microscopy for expression of MEKK1 and for positive TdT staining. The percentage of cells with apoptotic nuclei as determined by TdT staining for each condition was quantified in a blinded manner. At least 400 cells were counted for each condition in three separate experiments. B) HEK 293 cells expressing full length MEKK1 along with myr-Akt, wt-Akt, or p35 were lysed. Caspase activity was determined by measurement of production of a fluorescent cleavage product of a caspase 3 consensus substrate (DEVE-AFC), as described in the Material and Methods section. Levels of caspase activation are expressed as fold difference in comparison with cells expressing pCMV5 vector alone.

Fig. 6

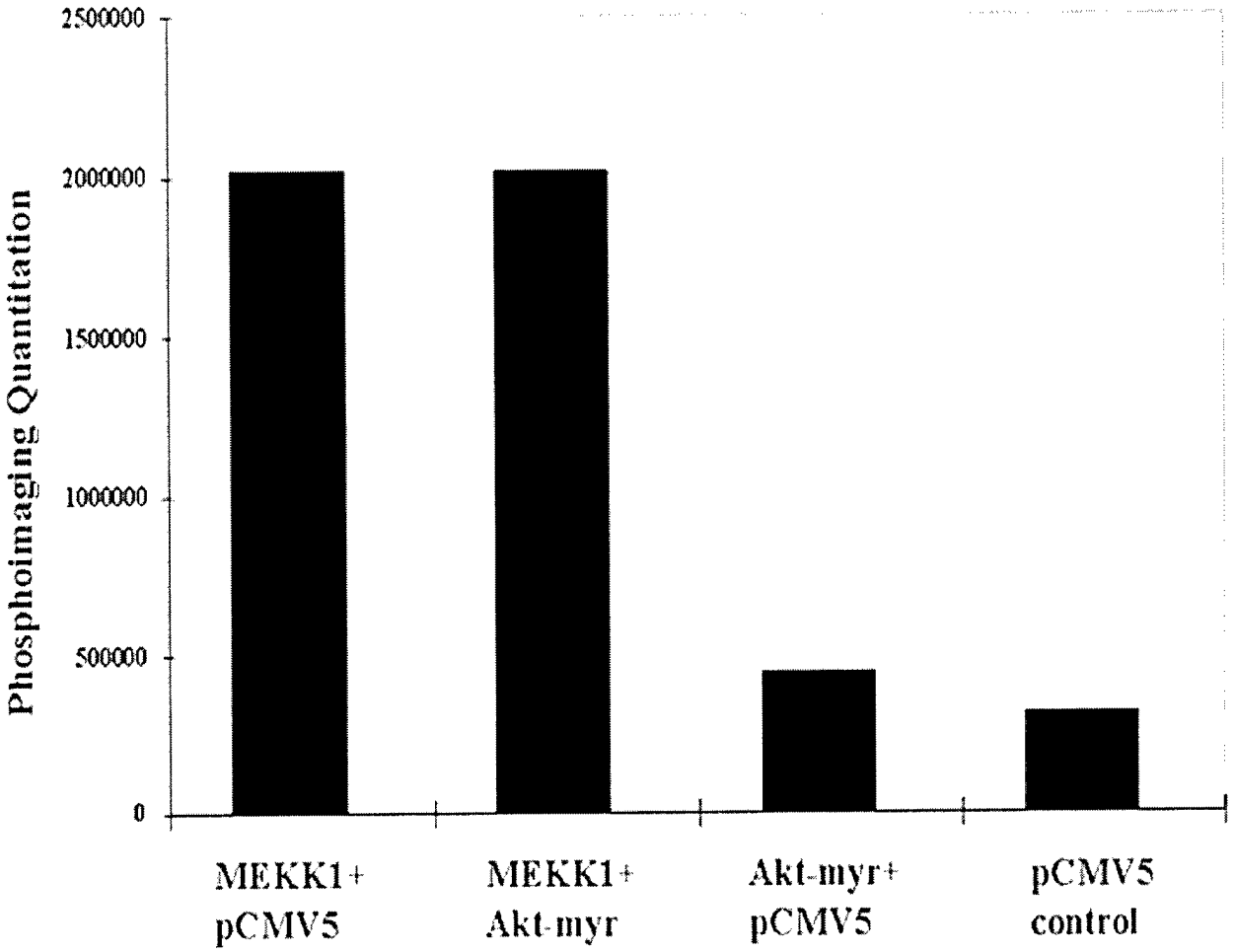

Figure 6 JNK activation following expression of MEKK1 in HEK293 cells. HEK 293 cells were transfected with MEKK1 or empty vector, along with either pCMV5 or myr-Akt, and analyzed for JNK activity in a GST-c-Jun assay. Kinase activity was quantified by phosphorimaging analysis.

Figure 7

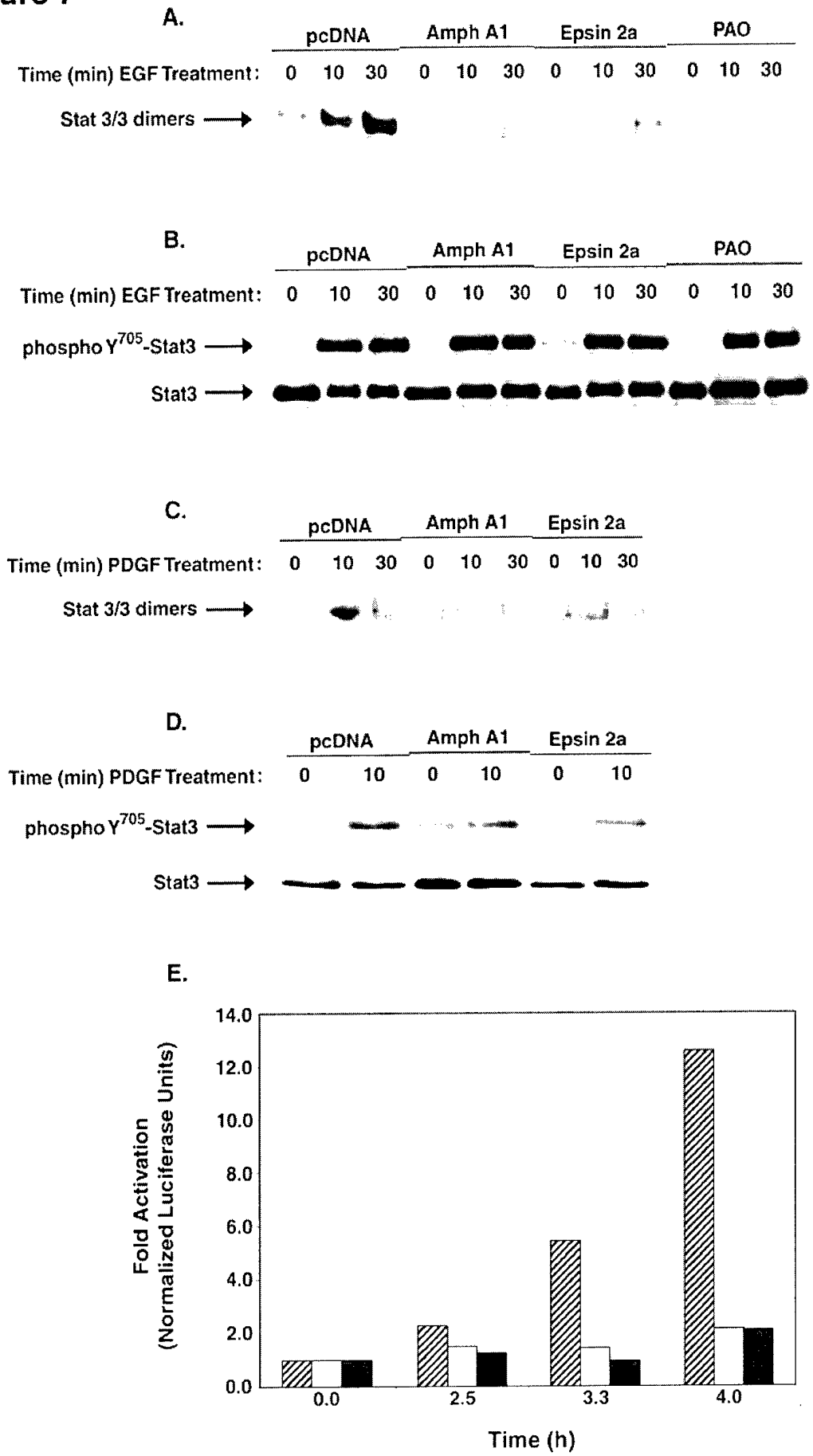
A.
pcDNA
Amph A1
Epsin 2a
PAO
Time (min) EGF Treatment: 0 10 30 0 10 30 0 10 30 0 10 30
Stat 3/3 dimers
B.
pcDNA
Amph A1
Epsin 2a
PAO
Time (min) EGF Treatment: 0 10 30 0 10 30 0 10 30 0 10 30
phospho Y705-Stat3
Stat3
C.
pcDNA
Amph A1
Epsin 2a
Time (min) PDGF Treatment: 0 10 30 0 10 30 0 10 30
Stat 3/3 dimers
D.
pcDNA
Amph A1
Epsin 2a
Time (min) PDGF Treatment: 0 10 0 10 0 10
phospho Y705-Stat3
Stat3
E.
Fold Activation (Normalized Luciferase Units)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0.0
2.5
3.3
4.0
Time (h)

Figure 7 **Nuclear Stat3 DNA-binding activity and Stat3-mediated gene regulation are inhibited by blocking endocytosis**. **(A,C)** DNA-binding activity of Stat3 complexes in either EGF-treated NIH-3T3/EGF-R (A) or PDGF-BB-treated Balb/c-3T3 (C) cells transfected with empty vector control (pcDNA) or the endocytosis inhibitors Amph A1 or Epsin 2a. **(B,D)** Western blot analysis was performed using antibodies to phosphoY^{705}-Stat3 or total Stat3 protein to probe immunoprecipitates prepared with antibodies to total Stat3 protein from lysates of either EGF-treated NIH-3T3/EGF-R (B) or PDGF-BB-treated Balb/c-3T3 (D) cells. **(E)** Stat3-dependent reporter gene expression. Balb/c-3T3 cells were transfected with control empty vector DNA (diagonal hatch) or expression vectors encoding the endocytosis inhibitors Amph A1 (white) or Epsin 2a (black), the Stat3 luciferase reporter construct pLucTKS3, and β-galactosidase vector as an internal control for transfection efficiency. The transfected cells were harvested 48 h post-transfection following serum starvation overnight and treatment with 50 ng/ml PDGF for the times indicated. Results are shown as fold luciferase activities normalized to the β-gal internal control. All data shown are representative of at least three independent experiments.

Fig. 8

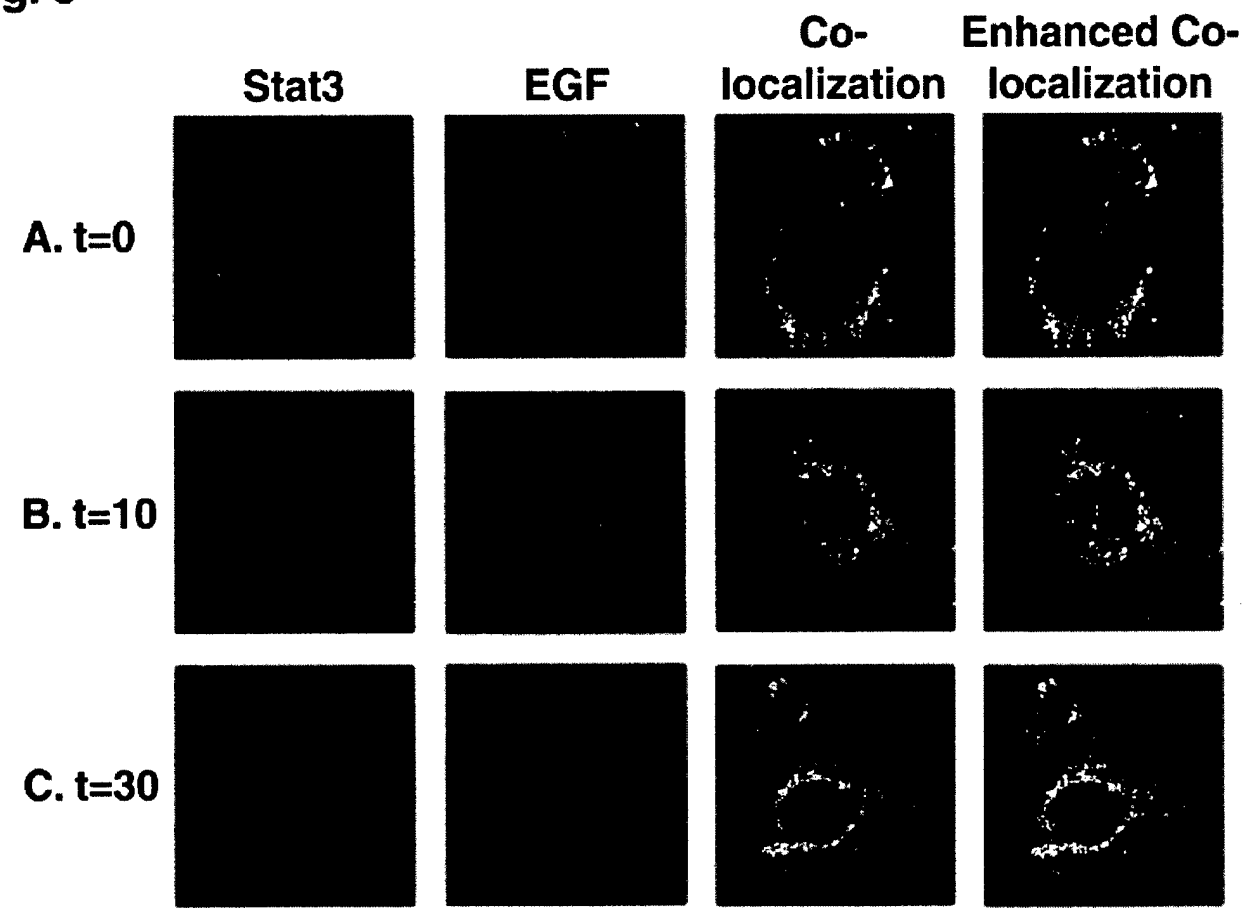

Figure 8 **Stat3 localizes sequentially to endocytic vesicles at the cell membrane (A), in the cytosol (B), or at the perinuclear region (C) following PDGF treatment.** **(A-C)** NIH-3T3 cells treated with 50 ng/ml PDGF for 45 min at 4°C to recruit ligand-bound receptors at the cell surface, and then warmed to 37°C for times indicated to enable endocytosis. Staining with rabbit anti-Stat3 (red) or mouse anti-AP-2 (green) antibodies detect Stat3 in endosomes following growth factor stimulation. Images were collected using the LSM 510 program on a Zeiss confocal microscope.

Fig. 9

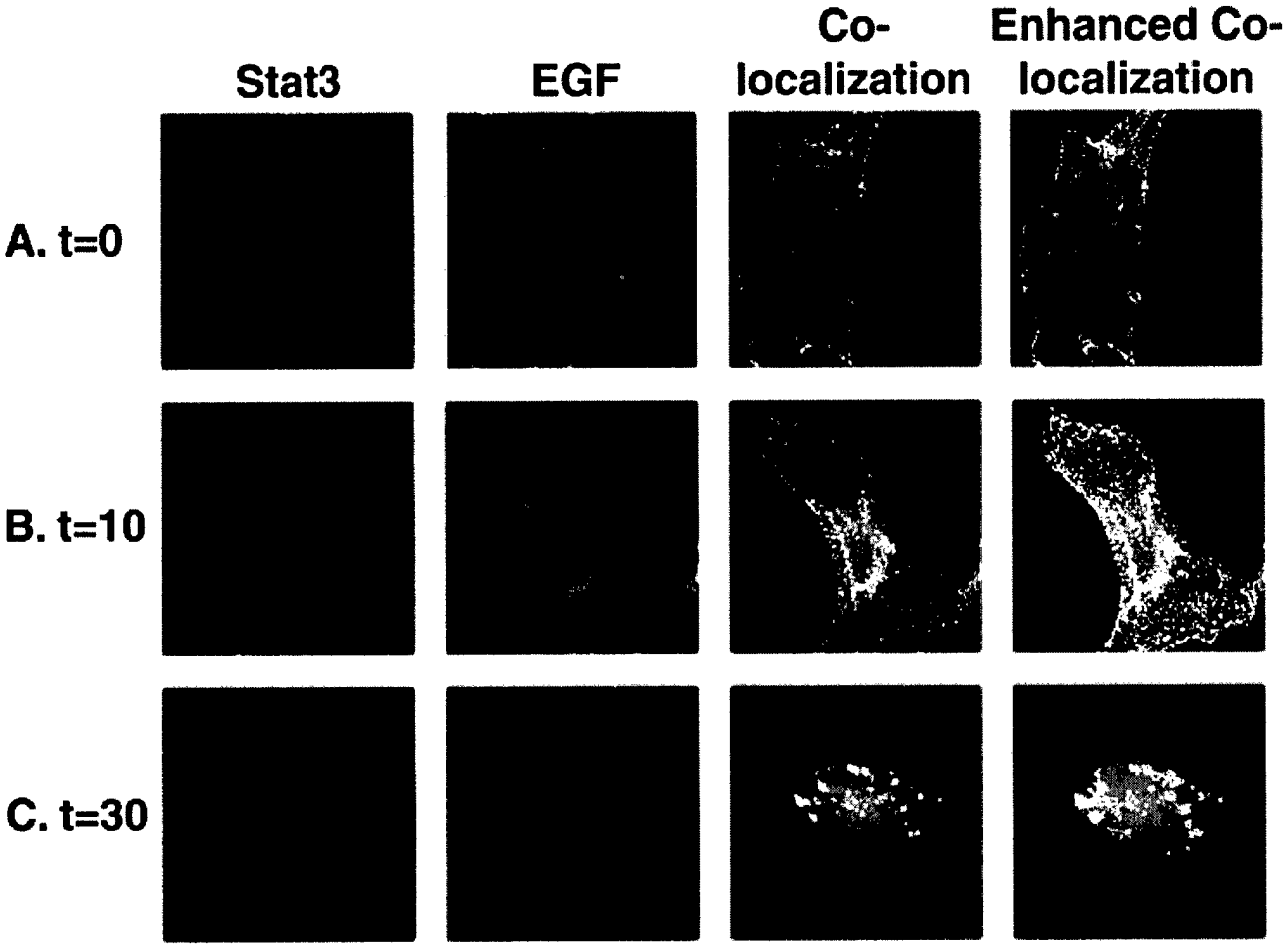

Figure 9 **Stat3 co-localizes with Alexa Fluor EGF sequentially at the cell membrane (A), in the cytoplasm (B) or the perinuclear region (C) following growth factor stimulation. (A-C)** NIH-3T3/EGF-R expressing Stat3 were treated with 2 μg/ml Alexa Fluor EGF for 45 min at 4°C to recruit ligand-bound receptor at the cell surface, and then warmed to 37°C for times indicated to enable endocytosis. Immunofluorescent staining with rabbit anti-Stat3 antibodies was performed as in Fig. 8. Images were collected using the LSM 510 program on a Zeiss confocal microscope. Live cell fluorescence also demonstrated extensive co-localization of Stat3-RFP and EGF-R-GFP following EGF treatment (results not shown).

Fig. 10

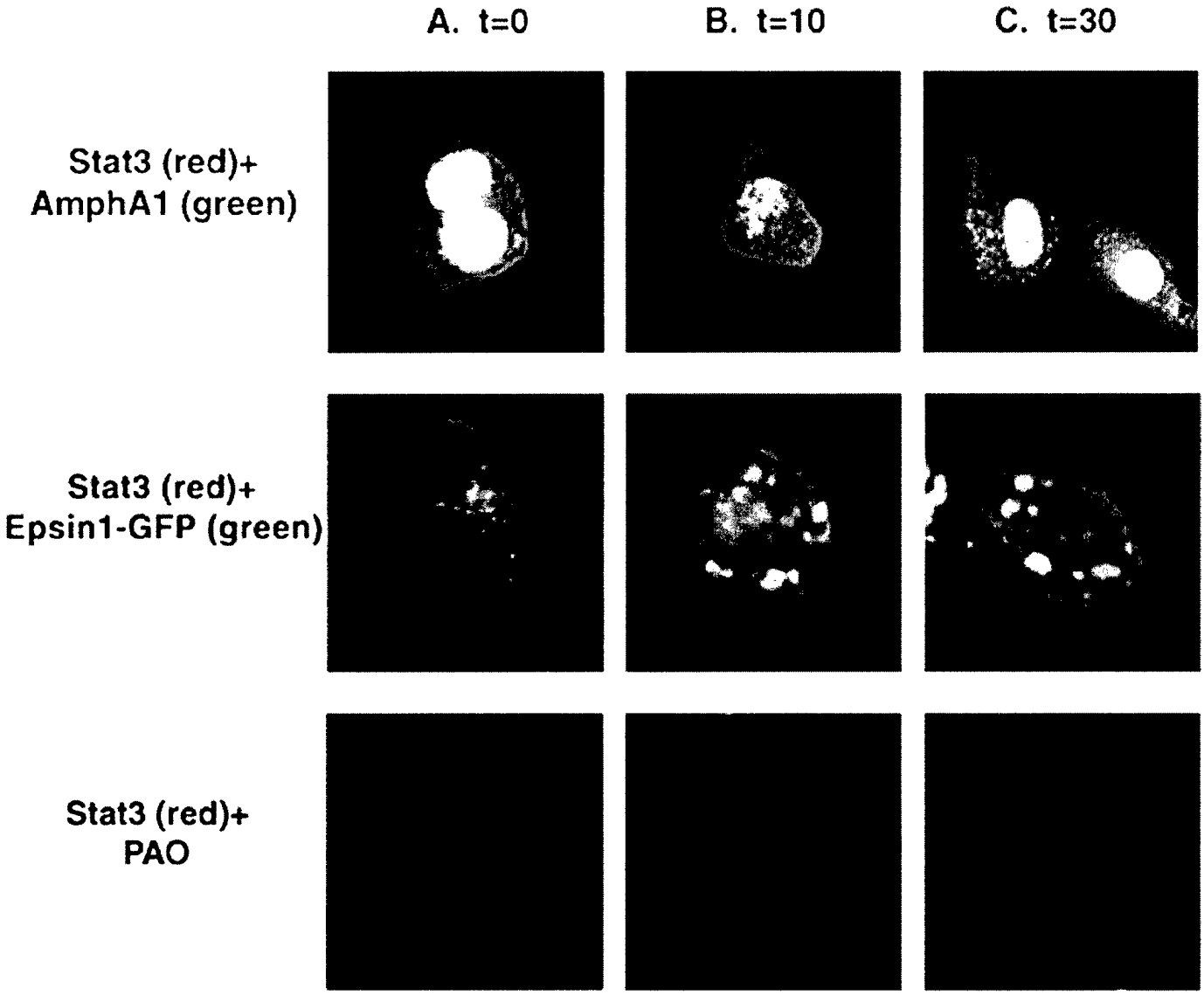

Figure 10 **Inhibition of endocytosis blocks Stat3 translocation to the perinuclear region.** Immunofluorescence analysis of NIH-3T3/EGF-R cells was carried out as described above after transfection of expression vectors encoding Stat3, Amph A1, or Epsin 2a-GFP. Antibodies to Stat3 or the HA-tag of Amph A1 were used to detect localization of these proteins. Transfected cells were treated with 1 μg/ml EGF for 0 min **(A)**, 10 min **(B)**, and 30 min **(C)**. Localization of Stat3 (red) and Amph A1 (green) or Epsin 2a-GFP (green) was analyzed using a Zeiss confocal microscope.

Fig. 11

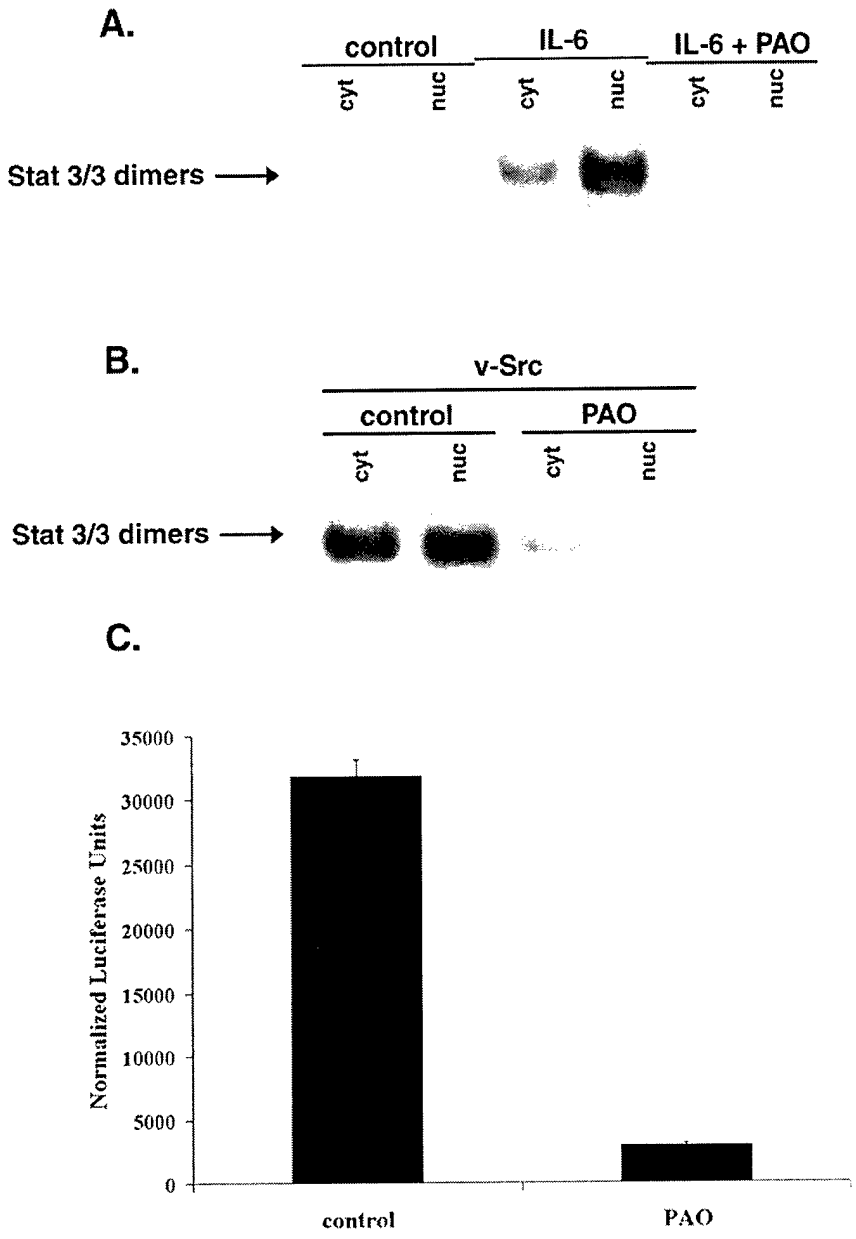

Figure 11 **Inhibition of endocytosis blocks v-Src and IL-6 activation and transcriptional activity.** **(A)** DNA-binding activity of either cytoplasmic (cyt) or nuclear (nuc) Stat3 complexes in control NIH-3T3 cells or cells treated with IL-6 with or without PAO treatment as described above. **(B)** DNA-binding activity of either cyt or nuc Stat3 complexes in NIH-3T3/v-Src cells. **(C)** Stat3-dependent reporter gene expression. NIH-3T3/v-Src cells with stable expression of the Stat3 luciferase reporter construct pLucTKS3, and β-galactosidase vector (as an internal control for transfection efficiency) were treated or not with PAO and Stat3 transcriptional activity was quantitated as above .